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BONE FORMATION IN VIVO: COMPARISON OF OSTEOGENESIS BY TRANSPLANTED MOUSE AND HUMAN MARROW STROMAL FIBROBLASTS

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Background. **Marrow stromal fibroblasts (MSFs) are known to contain bone precursor cells. However, the osteogenic potential of human MSFs has been poorly characterized. The aim of this study was to compare the osteogenic capacity of mouse and human MSFs after implantation in vivo.**

Methods. **After in vitro expansion, MSFs were loaded into a number of different vehicles and transplanted subcutaneously into immunodeficient mice.**

Results. **Mouse MSFs transplanted within gelatin, polyvinyl sponges, and collagen matrices all formed a capsule of cortical-like bone surrounding a cavity with active hematopoiesis. In transplants of MSFs from transgenic mice harboring type I procollagen-chloramphenicol acetyltransferase constructs, chloramphenicol acetyltransferase activity was maintained for up to 14 weeks, indicating prolonged bone formation by transplanted MSFs. New bone formation by human MSFs was more dependent on both the in vitro expansion conditions and transplantation vehicles. Within gelatin, woven bone was observed sporadically and only after culture in the presence of dexamethasone and L-ascorbic acid phosphate magnesium salt n-hydrate. Consistent bone formation by human MSFs was achieved only within vehicles containing hydroxyapatite/tricalcium phosphate ceramics (HA/TCP) in the form of blocks, powder, and HA/TCP powder-type I bovine fibrillar collagen strips, and bone was maintained for at least 19 weeks. Cells of the new bone were positive for human osteonectin showing their donor origin. HA/TCP powder, the HA/TCP powder-type I bovine fibrillar collagen strips, and HA/TCP powder held together with fibrin were easier to load and supported more extensive osteogenesis than HA/TCP blocks and thus may be more applicable for therapeutic use.**

Conclusions. **In this article, we describe the differences in the requirements for mouse and human MSFs to form bone, and report the development of a methodology for the consistent in vivo generation of extensive bone from human MSFs.**

Bone marrow fragments or cell suspensions of mouse, rat, guinea pig, rabbit, porcine, and canine origin form osteogenic tissue when transplanted into heterotopic sites in vivo. In closed systems, such as diffusion chambers, bone marrow constituents form bone, or bone and cartilage, depending on the size of the chamber. In an open system, such as under the kidney capsule where neovascularization can occur, bone ossicles surround a hematopoietic marrow, which results in the formation of a bone marrow organ (*1–6*). Bone marrow organs are characterized by the property of self-maintenance, that is, they provide physiological support to the hematopoietic tissue localized within them, and remain vital for the lifetime of the recipient animal (*2, 7*). In contrast, bone induced by either transitional epithelium of urinary bladder or demineralized bone matrix is not self-maintained in heterotopic sites without the continuous presence of an inducer (*8, 9*).

Marrow stromal fibroblasts (MSFs*) become the predominant adherent cell type when bone marrow is cultured in vitro (*10–12*). In cultures generated from single-cell suspensions of marrow, MSFs grow in colonies, each derived from a single precursor cell called the colony-forming unit-fibroblast (*13–15*). In addition to their fibroblast-like morphology, MSFs share a variety of fibroblastic features, but lack the basic characteristics of endothelial cells and macrophages (*16–19*). After extended culture, MSFs of mouse, rat, guinea pig, and rabbit origin have been reported to maintain the ability to form at least five types of connective tissue in transplantation systems. These tissues include bone, cartilage, fibrous tissue, adipose tissue, and hematopoiesis-supporting reticular stroma (*1, 12, 15, 20–24*). Thus, MSF populations contain pluripotent stromal stem cells that are capable of proliferation, renewal, and differentiation into several phenotypes (*12*). These stromal stem cells give rise to lineages distinct from that of hematopoietic stem cells (*13,*

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* Abbreviations: αMEM, α-modified minimum essential medium; AscP, L-ascorbic acid phosphate magnesium salt n-hydrate; CAT, chloramphenicol acetyltransferase; DBM, human demineralized bone matrix; Dex, dexamethasone; FBS, fetal bovine serum; HA/TCP, hydroxyapatite/tricalcium phosphate ceramics; HBSS, Hanks' balance salt solution; MSF, marrow stromal fibroblast.

25, 26). More mature osteoblastic cells isolated from rodent bone, such as calvariae, lose the stem cell properties of MSFs and lack the ability to form bone when transplanted after long-term culture. After short-term culture, they form bone, but not a bone marrow organ or cartilage (27, 28).

The osteogenic potential of human bone cells has also been studied using several experimental models. Primary bone cells derived from children occasionally form bone and cartilage, but do not support hematopoiesis after intramuscular transplantation into cortisone-pretreated mice (29). When primary bone cells are transplanted within diffusion chambers, bone formation can be achieved after cultivation in the presence of osteogenic inducers, but not after cultivation without them (30, 31). It has been recently reported that when viable fragments of human bone are transplanted into scid mice pretreated with radiation, osteoblasts survive and deposit new bone upon the preexisting bone fragments (32).

In contrast to rodent marrow cells, the osteogenic potential of human bone marrow cells is less well characterized. Previous reports indicate that when adult human marrow cells are transplanted in diffusion chambers, only unmineralized fibrous tissue is formed; however, marrow cells from young children occasionally develop osteogenic tissue (33). Like human bone cells, human MSFs have been reported to show no signs of osteogenesis in diffusion chambers transplanted intraperitoneally into nude mice (10, 31, 34), unless they had been cultured in the presence of osteogenic inducers (31). To date, there have been only two reports of bone formation in vivo by adult human cells of marrow origin (31, 34).

In this study, we have compared the osteogenic potential of mouse and human MSFs and further characterized the ability of different vehicles to support osteogenesis. Both mouse and human MSFs maintain their osteogenic potential after several passages in vitro. However, different in vitro culture conditions and transplant vehicles were found to be required by mouse and human MSFs in order to maintain and display their osteogenic capacities.

MATERIALS AND METHODS

Preparation of bone marrow and spleen cell suspensions. Eight- to 14-week-old transgenic mice carrying procollagen type I-chloramphenicol acetyltransferase (CAT) constructs (35) or nontransgenic control C57BL/6 mice were used to derive mouse MSFs. Bone marrow from the femoral, tibial, and humeral medullary cavities was flushed with α-modified minimum essential medium (αMEM; Life Technologies, Grand Island, NY). For comparative purposes, cells from mouse spleens were prepared by dissecting the spleen parenchyma and mincing the tissue. All procedures were performed in accordance to specifications of an approved small animal protocol (114–93).

Normal human bone fragments were obtained from the femoral neck or ileum of patients ranging from 5 to 11 years of age (two boys and eight girls; mean age, 8.5 years) undergoing corrective surgery under institutional review board-approved procedures. Fragments of trabecular bone with red marrow were scraped with a steel blade into αMEM and pipetted repeatedly to release the marrow cells. Bone marrow aspirates from the iliac crests of healthy volunteers (seven men and six women, 25–53 years old; mean age, 38.7 years) were collected with informed consent, using local anesthesia. All human samples were collected in accordance with National Institutes of Health regulations governing the use of human subjects under protocol D-0188. Aspirates were placed in ice-cold αMEM with 100 U/ml sodium heparin (Fisher Scientific, Fair Lawn, NJ) and centrifuged at 1000 rpm for 10 min; the cell pellet was resuspended in fresh αMEM. All preparations were pipetted repeatedly to break up cell aggregates. Subsequently, marrow and spleen cell suspensions were passed consecutively through 16- and 20-gauge needles before culture.

Fibroblast cell cultures. Human foreskin fibroblasts were cultured in Dulbecco's modified Eagle's medium (Life Technologies) containing 2 mM glutamine, 50 μg/ml streptomycin, 50 U/ml penicillin, and 10% fetal bovine serum (FBS; HyClone, Logan, UT). The cells were isolated and kindly provided by Mark DeNichilo and were used at the third cell passage.

High-density bone marrow and spleen cell cultures. Cells were plated at the following densities: for mouse marrow, the entire marrow content of six bones (two femora, two tibiae, two humeri), which contained $6–8\times10^7$ nucleated cells; for mouse spleen, $10–20\times10^7$ nucleated cells; for human surgical specimens, 5×10^6 to 5×10^7 nucleated cells; for human aspirates, 5×10^6 to 20×10^7 nucleated cells per flask. Cells were cultured in 75-cm^2 culture flasks (Becton Dickinson, Franklin Lakes, NJ) in 30 ml of growth medium, which was composed of αMEM, 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin sulfate (Biofluids, Rockville, MD), and 20% of preselected FBS (Life Technologies, or Atlanta Biological, Norcross, GA). Cells were cultured at 37°C in an atmosphere of 100% humidity and 5% CO_2. Where indicated, MSFs were cultured in growth medium supplemented with 10^{-8} M dexamethasone (Dex; Sigma, St. Louis, MO) and 10^{-4} M L-ascorbic acid phosphate magnesium salt n-hydrate (AscP; Wako, Osaka, Japan). Medium was replaced on day 1 for human aspirate cultures and on days 7 and 14 for all cultures, if not passaged by day 14.

The resulting adherent mouse cells were harvested using the following protocol: (1) two washes with Hanks' balanced salt solution (HBSS; Life Technologies); (2) incubation with chondroitinase ABC (20 mU/ml; Seikaguku Corp., Tokyo, Japan) in αMEM for 25 to 35 min at 37°C; (3) one wash with HBSS; (4) incubation with 1x trypsin-EDTA (Life Technologies) for 25 to 30 min at room temperature; (5) a second incubation with trypsin-EDTA for 25 to 30 min at 37°C; and (6) a final wash with growth medium. Steps 2 and 3 were omitted for passages greater than two. Human adherent cells were washed twice with HBSS and were treated with two consecutive applications of trypsin-EDTA for 10 to 15 min each at room temperature, followed by a wash with growth medium. Cold serum was added to the cells, and the portions were combined, vigorously pipetted, centrifuged, and resuspended in a fresh growth medium. The passaged cells were plated at 2×10^6 cells per 75-cm^2 flask. Some human cells were cryopreserved in a freezing medium consisting of αMEM (44%), FBS (50%), penicillin (100 U/ml), streptomycin sulfate (100 mg/ml), and dimethyl sulfoxide (5%; Sigma) and were stored in liquid nitrogen. After 1–15 months, the cells were thawed, plated, and passaged once or twice before transplantation.

Morphological and cytochemical analyses were performed on the marrow stromal cell populations before transplantation. Mouse cells from the 2nd and 10th passage, and human cells from the 3rd and 5th passage, were plated into two-well chamber slides (VWR Scientific, West Chester, PA) at 5×10^4 cells/well. After 24 hr, the presence of α-naphthyl acetate esterase and acid phosphatase (Sigma kits 91-A and 387-A, respectively) was determined. The number of positive and negative cells for both enzymes was determined by counting at least 200 cells in each of five different regions of the slides. In parallel experiments, the same cell populations were analyzed by flow cytometry using forward scatter versus side scatter with a FACScan (Becton Dickinson, Mansfield, MA).

Loading cells into the transplantation vehicles. Mouse MSFs (passages 1–10, $3.0–8.5\times10^6$ cells), mouse spleen cells (passages 2–3, $4–5\times10^6$ cells), human MSFs (passages 2–4, $1.5–16.0\times10^6$ cells), and human foreskin fibroblasts (passage 3, 3×10^6 cells) were loaded into the vehicles. After centrifugation at 1000 rpm for 10 min, the cell pellets were resuspended in 30–100 μl of growth medium. The following primary transplantation vehicles were loaded with cells: gelatin (Gelfoam, Upjohn, Kalamazoo, MI); polyvinyl sponges; porous

collagen matrices (American Biomaterials Corp., Princeton, NJ); poly(L-lactic acid) (Zimmer, Warsaw, IN), human demineralized bone matrix (DBM), particles 100–200 mm, to coarse, 1–4 mm (LifeNet Transplant Services, Virginia Beach, VA); hydroxyapatite/tricalcium phosphate ceramics (HA/TCP), blocks or powder; and HA/TCP powder-type I bovine fibrillar collagen strips (Collagraft strips, Zimmer). Approximate volumes of the vehicles used for transplantation, as well as methods of loading the vehicles with cells, are described in Table 1. Cells were loaded into HA/TCP blocks by a slight vacuum suction. The cell suspension was placed dropwise onto a block, and fluid was drawn through the block by touching the opposite side of the block with a Pasteur pipette connected to a vacuum pump.

A secondary vehicle was used in some experiments to organize HA/TCP or fine DBM powder particles with cells attached to them. Particles bearing cells were loaded into gelatin as described above. Alternatively, vehicle particles with adherent cells were bound together in collagen gels or fibrin clots (0.3–0.5 ml volume). The collagen gel was prepared according to manufacturer's (Zimmer) recommendations. The fibrin clots were prepared from mouse fibrinogen complex (American Red Cross, Rockville, MD, kindly provided by Dr. R. Ebert).

Transplantation procedures. Vehicles loaded with MSFs, mouse spleen fibroblasts, human foreskin fibroblasts, or empty vehicles were transplanted into mouse recipients. Three different strains of immunodeficient mice (8- to 15-week-old females) were used as subcutaneous transplant recipients (NIH-bg-nu-xidBr [beige], C.B.-17/IcrCrl-scidBr [scid], and C.B.-17/IcrCrl-scid-bgBr [scid/beige]), since the strain of mouse can potentially influence allogenic bone formation. Operations were performed under anesthesia achieved by intraperitoneal injection of 2.5% tribromoethanol (Sigma) at 0.018 ml/g body weight. Midlongitudinal skin incisions of about 1 cm in length were made on the dorsal surface of each mouse, and subcutaneous pockets were formed by blunt dissection. A single transplant was placed into each pocket, with up to four transplants per animal. The incisions were closed with surgical staples.

Fixation and histological examination of the transplants. The transplants were recovered at various time points between 2 and 19 weeks after transplantation and were fixed and partially decalcified for 2 days in Bouin's solution (Sigma). The transplants were then transferred to 70% ethanol until they were embedded in paraffin. After sectioning, sections were deparaffinized, hydrated, and stained with hematoxylin and eosin.

Immunostaining for human osteonectin. Immunohistochemical localization of human osteonectin was performed in human MSF transplants to identify the origin of the osteogenic tissue. After deparaffinization and rehydration, the sections were incubated in 0.13% pepsin and 0.01 N HCl for 1 hr at 37°C to reactivate the antigenicity of osteonectin. Indirect immunohistochemistry was carried out using rabbit anti-human osteonectin antibody (1:100) as the primary antibody (*36*). Goat anti-rabbit IgG antibody (1:200, Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used as secondary antibody. Mouse vertebral bone and femoral bone of human fetus were used for negative and positive controls, respectively. As another negative control, the primary antibody was substituted for normal rabbit serum (Vector Laboratories, Burlingame, CA).

Assay of mouse hematopoietic colony-forming cells. Mouse MSF transplants in gelatin sponges containing hematopoietic cells were harvested for their mononuclear cells. Cells were plated in duplicate at 1×10^5 cells/1 ml of murine methylcellulose medium (Terry Fox Laboratories, Vancouver, British Columbia, Canada) supplemented with 5% spleen-conditioned medium, 3 U/ml human erythropoietin, 50 ng/ml human interleukin 6, and 100 ng/ml rat stem cell factor (Amgen Inc., Thousand Oaks, CA). Colonies were counted at day 14.

Measurement of CAT activity. Transplants formed by mouse MSFs prepared from transgenic mice harboring ColCAT constructs were rinsed twice in HBSS and placed in 100 μl of extraction buffer (0.25 M Tris-HCl [pH 7.8] and 0.5% Triton X-100). Three consecutive freeze/thaw cycles (freezing on dry ice and thawing at 37°C for 1 min) were performed. The extracts were incubated at 65°C for 15 min to inactivate endogenous acetylase activity. CAT activity was quantitated by a fluor-diffusion assay using [^{3}H]acetyl coenzyme A (200 mCi/mmol; New England Nuclear, Boston, MA) at 0.1 μCi per assay. Cell extracts were overlaid with 5 ml of water-immiscible scintillation cocktail (Econofluor II, New England Nuclear). The samples were incubated at room temperature and aliquots were counted hourly. CAT activity was represented as the linear regression slope of the data plotted as cpm of product produced versus time of incubation and is expressed as cpm/transplant.

Low-density human marrow cell cultures. Because high-density cultures cannot be used to accurately determine the effect of culture conditions on MSF colony-forming efficiency or the rate of proliferation, low-density cultures were established to study the effect of Dex and AscP. Marrow single-cell suspensions were prepared from human surgical specimens by passing cells through needles of decreasing diameter (16, 20, and 23 gauges) and subsequently filtering cells through a cell strainer 2350 (Becton Dickinson, Franklin Lakes, NJ) to eliminate cell aggregates. Nucleated cells were plated at a density of 2×10^4 cells/cm^2. Two types of low-density cultures were used. In total cultures, the entire population (adherent and nonadherent) of plated marrow cells was left undisturbed until the time of harvest. To produce cultures of adherent cells only, cells were incubated for 2.5 hr at 37°C. Subsequently, unattached cells were aspirated, and cultures were washed vigorously four times with Dulbecco's modified Eagle's medium (Biofluids). The total and adherent cultures were

TABLE 1. Loading of MSFs into the transplantation vehicles[a]

Vehicle	Vehicle dimensions	Method of loading vehicles with cells	Loading results
Gelatin, polyvinyl sponge, and HA/TCP powder-bovine collagen strip	50–100-mm^3 cube blocks	Vehicles were wetted with growth medium, dried briefly between filter paper, and immediately placed into a dense cell suspension (volume was slightly less than the volume of the vehicle); cells were taken up as the vehicle expanded	>90% cells were incorporated into the vehicle
Collagen matrices and poly(L-lactic acid)	30–50-mm^3 square sheets		
DBM	25 mg of powder	Powder was washed in medium, mixed with cell suspensions, and incubated with rotation	>80% cells were incorporated into the vehicle
HA/TCP powder	40 mg of powder		
HA/TCP block	20–30-mm^3 disks	Cells were pumped into the disks by a slight vacuum suction	Not determined

[a] Vehicles with cells were incubated at 37°C for 40 to 90 min before transplantation.

used to determine whether Dex and AscP directly affected the adherent stromal population or whether the effect was mediated through the nonadherent population. Cells were cultured in the presence or absence of Dex and AscP. Half of the cultures were fixed on day 12 with methanol and stained with methyl violet (Sigma). MSF colonies composed of at least 50 cells were counted using a dissecting microscope, and colony-forming efficiency (number of colonies per 1×10^5 marrow cells plated) was determined. On day 14, the remaining cultures were washed twice with HBSS and harvested with trypsin-EDTA, and cell numbers were determined (Coulter Electronics, Hialeah, FL). Analysis of variance was performed and posttest comparison was completed using the Bonferroni multiple comparison test.

RESULTS

Marrow cell cultures. In high-density cultures of mouse and human marrow cells, the adherent cells reached confluence within 12 to 14 days. In mouse cultures, several morphological cell types were evident within the complex multilayered population, including fibroblast-like cells, fat cells, macrophages, and hematopoietic cells. After two passages, most cells exhibited a fibroblastic morphology consisting of a large flattened cytoplasm and a large oval nucleus with prominent nucleoli. These cells made up about 89% of the total adherent cell population. Using a FACScan with forward scatter to reflect the cell size, these cells had a relative forward scatter of 300–600 (Table 2). Cells in this fraction showed no detectable levels of α-naphthyl acetate esterase and acid phosphatase activity, which suggests that they represented MSFs (*17, 18, 37, 38*). The remaining cells showed high levels of α-naphthyl acetate esterase and acid phosphatase activity (Table 2). This smaller size fraction had a relative forward scatter of 50–200. These cells were round, bipolar, or stellate shaped and thus likely represented a mixture of macrophages and endothelial cells (*18, 37*). The percentage of these cells decreased with progressive passages (Table 2).

The human marrow primary cultures developed as discrete MSF colonies with varying morphology. The presence of cell types other than MSFs was substantially lower in the human cultures than in the mouse cultures, and no active hematopoiesis was evident. After three or five passages, adherent human marrow cells consisted of a nearly pure population of MSFs (Table 2).

Confirmation of donor origin and bone specificity of transplants. To confirm the origin of the cells associated with new bone formation, and to follow the fate of the transplanted mouse MSFs, MSFs were isolated from transgenic mice carrying type I procollagen reporter genes (ColCAT). ColCAT3.6, ColCAT2.3, and ColCAT1.7 contain 3520, 2296, and 1667 bp of the rat COL1A1 promoter, respectively (*35*). In both ColCAT3.6 and ColCAT2.3 transgenic mice, tissues expressing high levels of type I procollagen, such as bone, tendon, and teeth, exhibit high levels of the CAT reporter gene activity, whereas such tissues from ColCAT1.7 mice have undetectable levels of CAT activity. Thus, DNA elements between −2.3 and −1.7 kb are required for COL1A1 promoter expression in bone (*39*). When primary bone cells are isolated from transgenic calvariae and cultured in vitro, ColCAT3.6 cells retain promoter activity, whereas activity from ColCAT2.3 is lost, which suggests that the state of differentiation or the microenvironment of the osteoblastic cells determines which region of the promoter is utilized (*40*). Using the transgenic mouse MSFs as a model of osteogenesis, we followed the fate of MSFs by measuring CAT activity. MSFs from the transgenic mice formed bone in vivo, and both ColCAT3.6 and ColCAT2.3 displayed CAT activity that could be measured in the newly formed bone within gelatin sponges, polyvinyl sponges, and HA/TCP blocks. Using the procedures described in the *Materials and Methods*, we determined that CAT activity in 28-day transplants was 6761.7±1301.8 cpm and 2491.7±336.7 cpm per transplant for ColCAT3.6 and ColCAT2.3, respectively. These findings contrast with the observed promoter activity in primary cultures of MSFs, where CAT activity from both ColCAT3.6 and ColCAT2.3 was less that 35 cmp/hr/μg protein. This provides evidence that transgenic-derived MSFs: (1) populate the vehicle, (2) differentiate in vivo, and (3) reconstitute the activity of the 3.6-kb and 2.3-kb collagen I promoters.

As shown in Figure 1, anti-human osteonectin antibodies reacted with bone cells within the human MSF transplants. Intense immunostaining was found in both osteoblasts and osteocytes of the new bone, whereas no immunoreactivity above background levels was seen in the tissues surrounding the transplants. Thus, the osteogenic tissue formed by human MSF transplants was of human origin and was not formed by induced mouse cells.

Mouse MSF transplants. The sequence of events that takes place in heterotopic transplants of rodent bone marrow has been described in detail elsewhere (*2, 41*) and so will not be described here. In the present study, our aim was to compare the osteogenic potential of in vitro expanded mouse and human MSFs in different vehicles. The ability of different vehicles to support bone formation by mouse and human MSFs is summarized in Table 3.

Mouse MSF transplants in gelatin sponges formed new bone as early as 2 weeks after transplantation. The newly formed bone marrow organ consisted of a cortical-like structure surrounding an area of active hematopoiesis. The bone

TABLE 2. Characterization of cultured MSFs[a]

Source of MSFs	Passage	α-Naphthyl acetate esterase	Acid phosphatase	Smaller-size fraction[b]
Mouse	2	11.01±0.40	11.03±1.58	11.95
Mouse	10	4.77±0.64	5.86±0.76	2.92
Human	3	1.21±0.49	1.36±0.40	2.00
Human	5	1.55±0.36	0.97±0.55	5.27

[a] MSFs were assayed for α-naphthyl acetate esterase and acid phosphatase activity, and were size fractionated by FACScan to analyze the heterogeneity within the MSF cultures.

[b] Relative forward scatter of 50–200 as measured by FACScan, versus 300–600 for MSFs. The data represent the percent of smaller-size cells or the mean percent of cells positive for the respective enzymes ± SEM.

exhibited lacunae containing osteocytes as well as osteoblastic layers on both the inner and outer surfaces. Isolated osteoclasts were occasionally found adjacent to the bone.

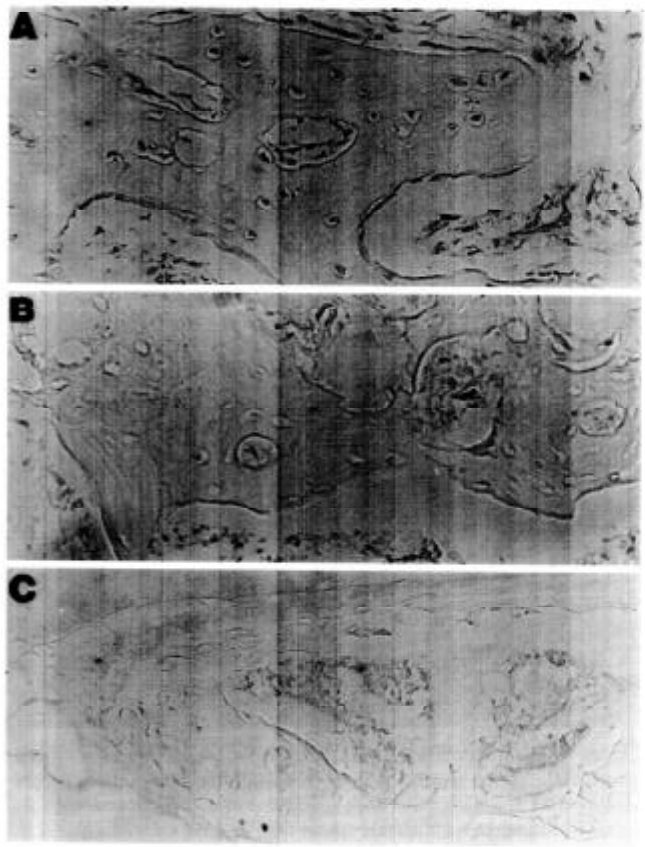

FIGURE 1. Confirmation of human origin of bone formed by transplants of human MSFs: (A) immunohistochemical staining in a transplant of human MSFs in the HA/TCP powder-type I bovine fibrillar collagen mixture recovered at 8 weeks after transplantation using antibodies raised against human osteonectin; (B) immunostaining with nonimmune rabbit antiserum; (C) immunostaining of mouse vertebral bone with anti-human osteonectin antibodies (×165).

Inside the bony capsule, between bone trabeculae and portions of vehicle undergoing resorption, bone marrow tissue, which included blood vessels and sinuses, reticular and fat stroma, and hematopoietic cells of all lineages and stages of maturation, was observed. With continued growth, the bone capsule became thicker and the hematopoietic tissue more abundant (Fig. 2, A and B). No signs of degeneration were observed in the heterotopic bone marrow organs for at least 14 weeks after transplantation. In four separate transplants recovered after 44 days, smears of the hematopoietic elements showed normal maturation with megakaryocytes. Methycellulose cultures counted at day 14 revealed 179.6 ± 15.0 colonies/10^5 cells. These colony numbers are equivalent to those seen when plating mononuclear cells from skeletal murine marrow under the same conditions.

Transplants of mouse MSFs in polyvinyl sponges and collagen matrices were similar in appearance to transplants in gelatin sponges. These transplants consisted of a thin capsule of cortical bone surrounding a cavity with bone trabeculae, vehicle remnants, blood vessels, fat cells, and hematopoietic tissue (Fig. 3, A and B). However, the thickness of the cortices, as well as the level of hematopoiesis, was not as pronounced as in gelatin vehicles.

Two weeks after transplantation of mouse MSFs in HA/TCP blocks, newly formed bone was observed in vehicle pores at the periphery of the transplants. Most of the internal pores contained fibrous tissue and vascular structures. After 4 to 5 weeks, many pores were filled with woven bone, which was deposited against the vehicle walls. New bone showed osteocytes embedded within the matrix, and a prominent osteoblastic layer was evident on luminal surfaces (Fig. 4). After 6 to 10 weeks, transplants showed areas of vehicle resorption and bone remodeling. The bone trabeculae began to merge on the periphery of the transplants, forming an incomplete bony capsule. Larger pores were layered with lamellar-like bone surrounding reticular and fat stroma with abundant hematopoietic tissue.

No obvious correlations were observed between the rate or the extent of bone formation and the number of transplanted MSFs (ranging from 3.0 to 8.5×10^6 cells per gelatin vehicle). However, no attempt was made to determine the lowest ratio

TABLE 3. Comparison of osteogenesis by MSFs in different transplantation vehicles[a]

Transplantation vehicle		Source of MSFs		
Primary vehicle	Secondary vehicle	Mouse	Human (Dex/AscP)	Human (w/o Dex/AscP)
Gelatin		21/23	5/28	0/30
Polyvinyl sponge		3/5	0/3	ND
Collagen matrix		2/2	ND	ND
HA/TCP block		10/10	13/14	3/3
Poly(L-lactic acid)			0/2	0/2
DBM			0/3	ND
DBM	Gelatin		0/9	0/8
DBM	Fibrin clot		0/15	ND
HA/TCP powder			13/15	10/10
HA/TCP powder	Gelatin		2/4	0/2
HA/TCP powder	Fibrin clot		12/12	ND
HA/TCP powder	Collagen gel		0/6	ND
HA/TCP powder-bovine collagen strip			20/23	10/10

[a] Values represent the number of transplants with new bone formation per total number of transplants. MSFs were cultured in growth medium in the presence or absence of Dex and AscP. ND, not determined.

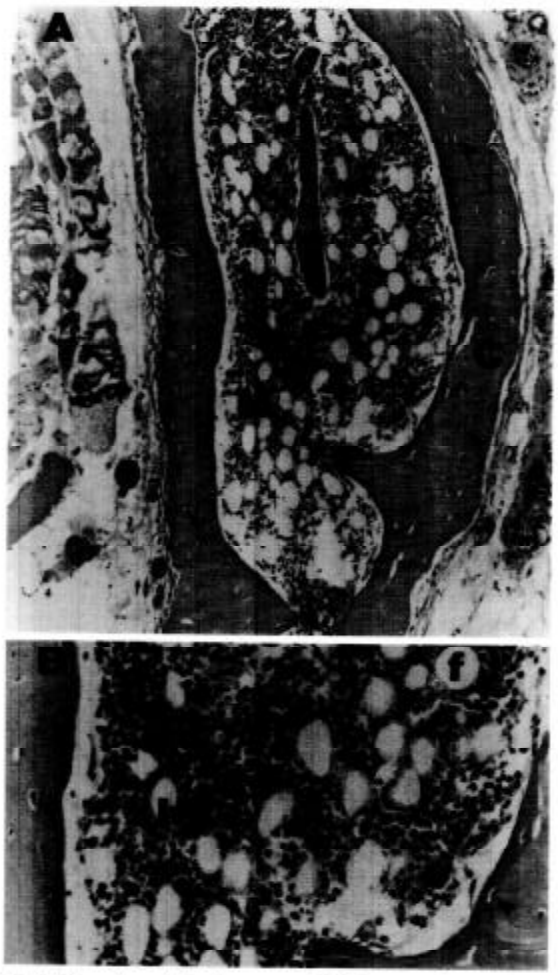

FIGURE 2. Bone formation by mouse MSFs transplanted within a gelatin sponge. The transplant was harvested at 14 weeks. New bone shows a cortical-like bone capsule (C) surrounding a bone marrow cavity (M) containing hematopoietic tissue (hp), fat cells (f), and trabecular bone (T). The arrowheads indicate embedded osteocytes (A: hematoxylin and eosin, ×82.5; B: hematoxylin and eosin, ×165).

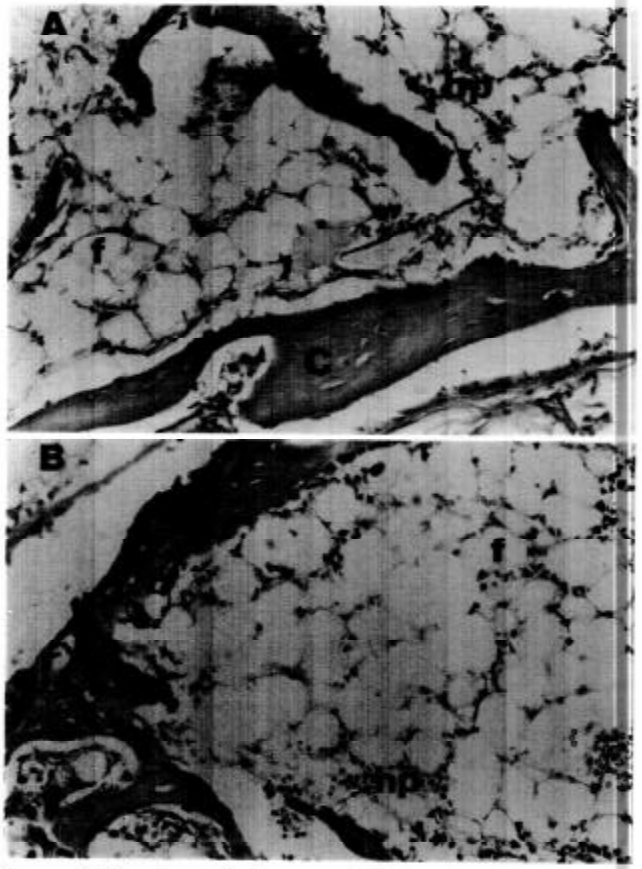

FIGURE 3. Bone formation by mouse MSFs transplanted within a collagen matrix (A) and a polyvinyl sponge (B). Transplants were harvested at 4 weeks. New bone consisted of a cortical-like capsule (C) surrounding hematopoietic tissue (hp) and fat cells (f) (A and B: hematoxylin and eosin, ×165).

of cells to vehicle needed for osteogenesis in this study. MSFs from in vitro passages 1–10 were capable of bone formation. In one experiment, MSFs from the 13th passage ceased to proliferate in vitro and failed to form bone in vivo. The presence of Dex and AscP in the growth medium did not influence the rate and extent of bone formation, or the abundance and morphological appearance of hematopoietic tissue. MSFs of both transgenic mice and nontransgenic C57BL/6 mice were capable of bone formation in both beige and scid immunodeficient recipient mice.

Bone formation was not observed in any control experiment that consisted of vehicles without cells. After several weeks in vivo, control transplants showed areas of soft connective tissue ingrowth, vehicle resorption, and connective tissue encapsulation. Abundant growth of fibrous tissue with no bone formation was observed in gelatin transplants of mouse spleen fibroblasts at 4–10 weeks. When mouse MSFs were transplanted into allogenic immunocompetent recipients, osteogenesis was not observed (data not shown).

Human MSF transplants. Between 1.5×10^6 and 1.6×10^7 human MSFs were transplanted within gelatin sponges. After 3 to 11 weeks, many transplants had been resorbed, and the majority of the remaining transplants contained fibrous tissue. However, bone was formed in a few transplants by human MSFs that had been cultured in the presence of Dex and AscP (Table 3). In those gelatin transplants that did form bone, a single trabecula of woven bone with no signs of remodeling was observed. The bone ossicle contained embedded osteocytes and was surrounded by a noncontinuous osteoblastic layer, but had no cavity and no hematopoietic tissue (Fig. 5A).

Human MSFs within HA/TCP-containing vehicles formed bone regardless of whether the cells had been cultured in the presence or absence of Dex and AscP (Table 3). In HA/TCP blocks containing human MSFs, bone was formed in all but one transplant (Table 3). At early stages (4–5 weeks), bone was found in several peripheral pores deposited against the walls of the vehicle, with an osteoblastic layer lining the luminal surface and osteocytes embedded within lacunae (Fig. 5B). After 6 to 10 weeks, the transplants showed areas of vehicle resorption, and the new bone acquired a more lamellar-like structure. Bone foci were larger and tended to

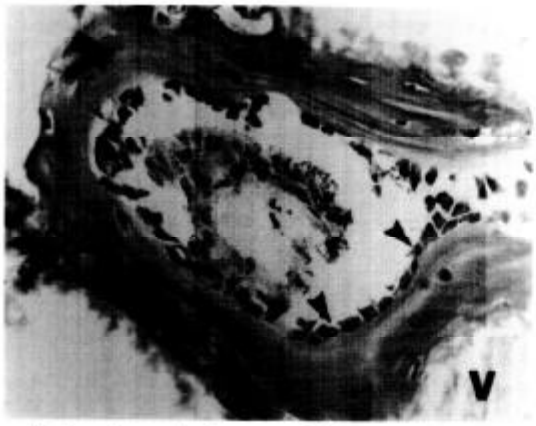

FIGURE 4. Bone formation by mouse MSFs transplanted within an HA/TCP block. The transplant was harvested at 5 weeks. Woven bone was deposited within the vehicle (V). Osteoblasts (large arrowheads) line the lumen surface and osteocytes (small arrowheads) are embedded within the bone matrix (hematoxylin and eosin, ×165).

merge, forming an incomplete capsule near the transplant surface.

Osteogenesis was observed in both HA/TCP powder and HA/TCP powder-bovine collagen strips (Table 3) and was more extensive than in HA/TCP blocks. By 4 to 6 weeks, numerous bone trabeculae with embedded osteocytes and prominent osteoblastic layers surrounded the vehicle particles. The new bone formed networks of interconnecting trabecular structures, with the intratrabecular space filled with fibrous tissue (Fig. 6A). After 8 to 10 weeks, the transplants contained an extensive network of bone (Fig. 6B). Degeneration was not detected in transplants of at least 19 weeks.

No bone formation was observed in transplants of human foreskin fibroblasts in either HA/TCP powder or HA/TCP powder-bovine collagen strips. In these control transplants fixed at 3–8 weeks, only fibrous tissue was observed. An additional control consisting of transplants of empty HA/TCP powder and HA/TCP-bovine collagen strip vehicles also showed only fibrous tissue growth (data not shown).

In many transplants of human MSFs in HA/TCP-containing vehicles, areas of hematopoietic tissue were observed. The extent of the hematopoietic tissue was usually dependent on the degree of bone formation. The hematopoietic cells were always closely associated with newly formed bone. These cells consisted of predominantly mature granulocytes, although some hematopoietic precursors may have been present (Fig. 7). The architecture of the ceramic vehicles precluded recovery of sufficient hematopoietic cell numbers to further characterize them.

As described above, in transplants using gelatin, human MSFs cultured in the presence of Dex and AscP infrequently formed bone, whereas human MSFs cultured without those supplements never formed bone (Table 3). Part of this difference may be due to stimulation of MSF proliferation by Dex and AscP. To further study the effect of Dex and AscP treatment of MSFs, the rate of proliferation of cells plated at low density in the presence or absence of Dex and AscP was studied in vitro. At low initial density plating, human MSFs grew as discrete colonies, with only a few macrophages apparent throughout the cultures. The combined action of Dex

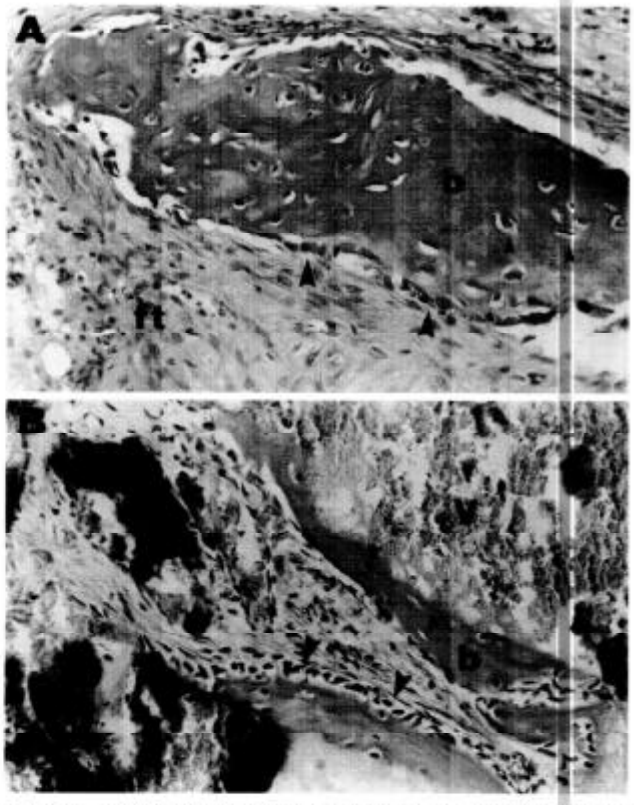

FIGURE 5. Bone formation by human MSFs. (A) The MSFs were cultured in the presence of Dex and AscP. After 8 weeks, new bone formed by human MSFs transplanted within gelatin sponges consisted of a single trabecula (b) surrounded by fibrous tissue (ft) with no marrow space. (B) Human MSFs transplanted within HA/TCP blocks formed bone within the vehicle pores (V) at 4 weeks. The large arrowheads designate osteoblasts. The small arrowheads designate osteocytes (hematoxylin and eosin, ×165).

and AscP did not change human MSF colony-forming efficiency (total number of colonies formed per 10^5 cells). However, the total cell number at the end of the culture period was elevated in the presence of Dex and AscP, which indicates an increase in the number of MSFs per colony (Fig. 8). This increase was statistically significant not only in total cultures (adherent and nonadherent cells), but also in adherent cultures (nonadherent cells removed after 2.5 hr), indicating a direct effect on MSFs rather that an effect mediated by the nonadherent population.

Human MSFs did not form bone when transplanted with human DBM. The transplants typically consisted of nonvital DBM particles surrounded by fibrous tissue. Likewise, only fibrous tissue was observed in transplants of MSFs within polyvinyl sponges and poly(L-lactic acid) vehicles (Table 3). The results of the human MSF transplantation studies revealed no obvious difference based on numbers of MSFs transplanted per vehicle, on strain of immunodeficient mouse recipients (beige, scid, or beige/scid mice), or on whether fresh or previously frozen MSFs were used. The source of bone marrow (surgical specimen versus marrow aspirate, donor age, and sex) had no obvious influence on bone formation (Table 4).

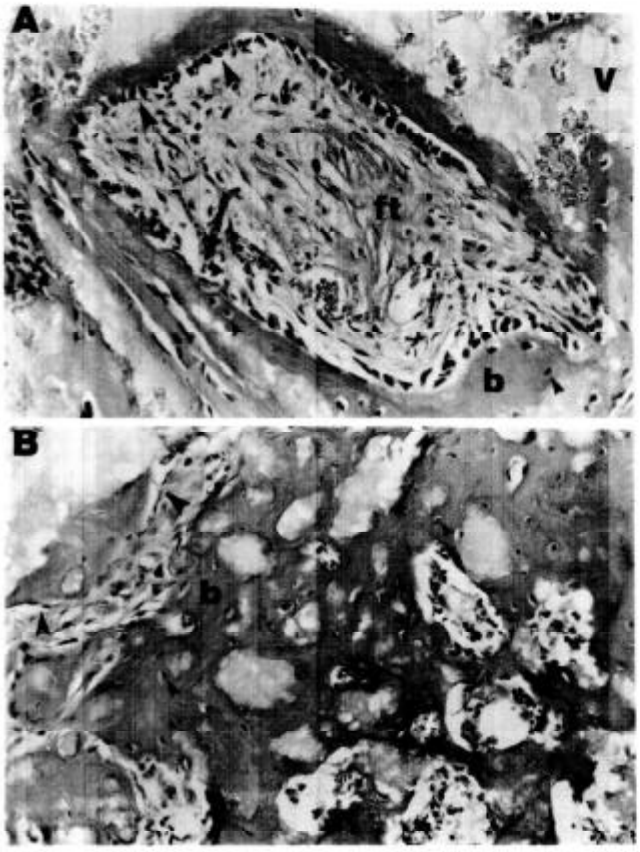

FIGURE 6. Bone formation by human MSFs transplanted within HA/TCP powder. (A) The transplant was harvested at 5 weeks. New bone (b) formed around the vehicle particles (V) and surrounded an area of fibrous tissue (ft). (B) Transplant of human MSFs in HA/TCP powder and gelatin sponge harvested at 8 weeks. Bone formed networks of interconnecting trabecular-like structures (b) with the intratrabecular spaces filled with fibrous tissue and partially resorbed vehicle matrix. The large arrowheads designate osteoblasts. The small arrowheads designate osteocytes (hematoxylin and eosin, ×165).

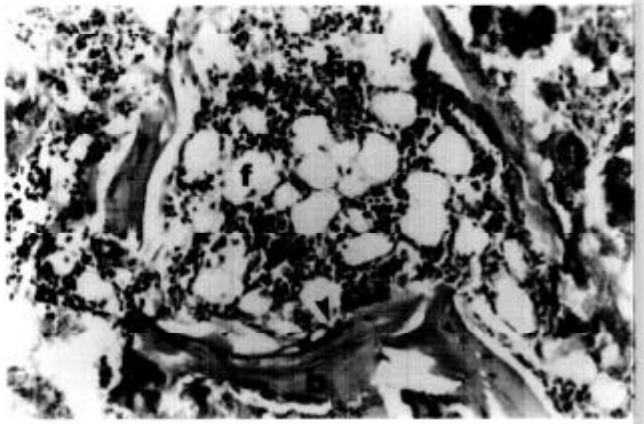

FIGURE 7. Bone formation by human MSFs transplanted within HA/TCP powder. The transplant was harvested at 8 weeks. New bone (b) surrounds a cavity with hematopoietic tissue (hp) and fat cells (f). The large arrowheads designate osteoblasts. The small arrowheads designate osteocytes (hematoxylin and eosin, ×165).

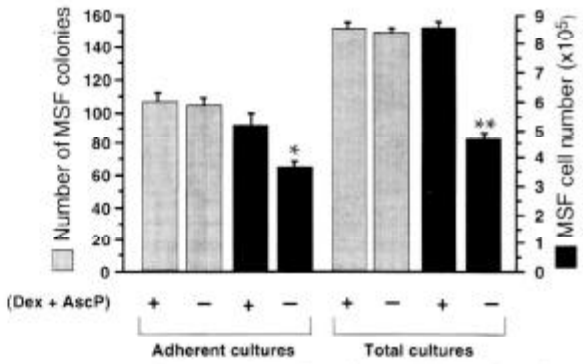

FIGURE 8. Effects of Dex and AscP on human MSF colony-forming efficiency and total MSF cell numbers in primary low-density marrow cell cultures. After 12 days, one half of the flasks were fixed and the number of MSF colonies was counted. After 14 days, MSFs from the remaining flasks were harvested and the cell number was determined. Values represent the mean ± SEM for three flasks. Shaded bars represent the number of MSF colonies. Solid bars represent MSF cell number. $^{*}P<0.01$; $^{**}P<0.001$.

The major goals of this study were to determine whether cultured MSFs could form bone within an open model system in vivo, to compare mouse and human models, and to test a range of different vehicle constructs. Because of the diverse range of implant materials compared in this study, the accurate quantitation of the bone tissue formed and the influence of donor age and marrow localization was not complete.

DISCUSSION

When bone marrow is transplanted into diffusion chambers, the transplanted cells are the only source of new bone (*1, 3, 4, 6*). However, when bone marrow is transplanted in an open system, the origin (recipient or donor) of the cells that make new bone needs to be established. Transplants of quail marrow into nude mice showed a biphasic switch in the origin of new bone cells. After 3 to 4 weeks in vivo, osteocytes were of donor origin, but were identified using a specific nuclear marker to be of host origin at 8–12 weeks (*42*). However, studies using chromosomal markers, mouse strain-specific antiserum, species-specific antibodies, and reverse transplantation have shown that in heterotopic transplants of rodent marrow cells, hematopoietic cells and macrophages originate from the recipient, whereas bone-forming cells are exclusively of donor origin, up to at least 40 weeks after transplantation (*7, 13, 43, 44*). In addition, transplantation of male mouse bone marrow to female mouse recipients, followed by retransplantation of the heterotopic bone marrow organ contents to female mice preimmunized against male antigens, has shown that no bone precursor cells of recipient origin appear in heterotopic transplants for at least 2.5–3 months in the primary recipient (*43*).

A previous study using human-specific antibodies showed the donor origin of bone in transplants of human MSFs (*34*). Our results using immunohistochemical localization with antibodies against human osteonectin also show that the new bone tissue in 8-week-old transplants of human MSFs is formed by the implanted human cells rather than by cells of the recipient mouse. Our data using the transgenic marker genes (ColCAT3.6 and ColCAT2.3) as markers for osteogen-

TABLE 4. Comparison of osteogenesis by human MSFs from surgical specimens and aspirates[a]

Transplantation vehicle		Source of human MSFs			
Primary vehicle	Secondary vehicle	Surgical specimens		Aspirates	
		Males	Females	Males	Females
Gelatin		1/9	2/22	2/27	
Polyvinyl sponge				0/3	
Poly(L-lactic acid)					0/4
DBM					0/3
DBM	Gelatin			0/5	0/12
DBM	Fibrin clot			0/3	0/12
HA/TCP block			3/3	5/6	8/8
HA/TCP powder		4/4	8/8	3/3	8/10
HA/TCP powder	Gelatin			2/6	
HA/TCP powder	Fibrin clot		8/8	2/2	2/2
HA/TCP powder	Collagen gel		0/2		0/4
HA/TCP powder-bovine collagen strip		4/4	8/8	3/3	15/18

[a] Values represent the number of transplants with new bone formation per total number of transplants. MSFs were obtained from normal bone fragments of 10 patients undergoing corrective surgery (two boys and eight girls; mean age: 8.5 years) and from bone marrow aspirates of 13 normal volunteers (seven men and six women; mean age: 38.7 years).

esis confirm the donor origin of new bone in mouse MSF heterotopic transplants. The donor origin of new bone in MSF heterotopic transplants is further supported by the absence of osteogenesis in the transplants of empty vehicles (or of those transplanted with mouse spleen fibroblasts and human foreskin fibroblasts) and in the transplants of mouse MSFs into allogeneic immunocompetent recipient mice. Taken together, these data suggest that in MSF transplants, new bone tissue sustains its donor origin and does not undergo substitution by bone tissue of recipient origin.

Since the adherent marrow cell culture represents a heterogeneous population of cells, a potential source of ambiguity is the exact nature of the cells responsible for new bone formation. The adherent population in mouse marrow cell cultures may include MSFs, fat cells, macrophages, endothelial cells, and some persistent hematopoietic progenitor cells (*14, 17, 18, 45*). In human marrow cell cultures, the adherent population is more homogenous, consisting of MSFs with only a small percentage of macrophages, which decreases during progressive passages (*25, 45, 46*). In the present study, initial characterization was performed on the adherent marrow cells before transplantation. Passaged human cells were composed of a nearly pure MSF population. Passaged mouse cells contained a substantial portion of macrophages and endothelial cells in addition to MSFs, but the percentage of these cells decreased with consecutive passages. It cannot be completely ruled out that in transplants of mouse cells, cell types other than MSFs contributed to the formation of new bone. However, the osteogenic capacity of these cells was not altered after several passages and was impaired only at the point when MSFs lost their proliferative ability. In addition, individual MSF colonies and single colony-derived strains of mouse, guinea pig, and rabbit origin have been shown to form bone upon transplantation in vivo (*15, 20, 22, 24*). Thus, these data suggest that in transplants of adherent marrow cells, MSFs are the cells most likely to form new bone.

Our results show that osteogenesis by MSFs can be influenced by the composition of the transplantation vehicle. Mouse MSFs formed bone within all vehicles that we tested. In contrast, consistent bone formation by human MSFs was achieved only within HA/TCP blocks, HA/TCP powder, and HA/TCP powder-bovine collagen strips. HA/TCP powder, HA/TCP powder-bovine collagen strips, and HA/TCP powder held together with fibrin were easier to load and supported more extensive osteogenesis than HA/TCP blocks and thus may be more applicable for therapeutic use. When gelatin was used as the primary vehicle, only a small number of human MSF transplants developed bone. No bone formation was observed with the polyvinyl sponge, poly(L-lactic acid), and human DBM vehicles. Thus, hydroxyapatite and/or tricalcium phosphate support the ability of human MSFs to form bone in vivo. MSFs expanded in vitro may already be committed to osteogenic differentiation, and the underlying mechanism may be "permissive" rather than "inductive."

A number of variables had no effect on bone formation in the transplants of mouse and human MSFs. In mouse, neither the marrow origin (C57BL/6 versus noninbred mice), the presence of Dex and AscP in culture medium, the number of in vitro passages before transplantation, the number of MSFs loaded per vehicle, the nature of transplantation vehicle, nor the phenotype of the immunodeficient recipient mice had any influence on bone formation. The factors that did not influence osteogenesis by human MSFs included marrow origin (age and sex of donor, skeletal location of excised bone, and method of marrow preparation), number of in vitro passages before transplantation, freezing of MSFs, number of MSFs loaded per vehicle, and phenotype of the immunodeficient recipient mice. In a previous study, bone was formed only when more than 30,000 rat MSFs were transplanted per vehicle (27 mm^3) (*47*). In our study, MSF cell number per a unit vehicle volume exceeded this value. Since our goal was to determine whether a vehicle supported bone formation, we did not titrate the number of MSFs needed per unit vehicle volume. The observation that the number of in vitro passages was not a critical factor suggests that a sufficient number of MSFs maintain their osteogenic and proliferative properties throughout the in vitro culture period examined.

Culture in the presence of Dex and AscP did not influence osteogenesis by mouse MSFs or by human MSFs transplanted within HA/TCP-containing vehicles. However, if human MSFs were transplanted within gelatin sponges, they

formed bone only after culture in the presence of Dex and AscP. These results are consistent with the results of Gundle and co-workers (*31*), who showed that human MSFs formed bone after cultivation with and without Dex and AscP in HA/TCP-containing transplants. In contrast, human MSF transplants within diffusion chambers formed bone only when cultured with Dex and AscP (*31*). Taken together, both findings imply that cultivation of MSFs with Dex and AscP is required for osteogenesis in "nonsupportive" vehicles, but is not essential for osteogenesis in "supportive" (HA/TCP) vehicles.

Previously, Dex and ascorbate have been shown to stimulate osteogenic differentiation of MSFs in vitro (*6*, *48–54*). In the present study, the combined action of Dex and AscP stimulated the rate of in vitro proliferation of human MSFs without increasing colony numbers (Fig. 8). This stimulation does not require the presence of hematopoietic cells and likely occurs by direct action on MSFs. It is not clear, therefore, whether the positive effect of Dex and AscP on bone formation by human MSFs in nonsupportive vehicles in vivo is caused by in vitro stimulation of proliferation, osteogenic differentiation, or both. Interestingly, proliferation of rat MSFs was decreased by 10^{-8} M Dex (*53*), which indicates that the effect of Dex may be species specific.

The relationship between bone formation and hematopoiesis is well established. However, osteogenesis without the generation of a hematopoietic microenvironment has been described (*20*, *55*). These studies suggest that bone without hematopoiesis is formed by stromal precursor cells that have lost their multipotentiality and are committed only to osteogenic differentiation. Our results suggest that the relationship between osteogenesis and a hematopoietic microenvironment may be more complex. The same populations of human MSFs formed bone with or without accompanying hematopoietic tissue depending on the transplantation conditions.

These studies have begun to characterize the in vitro manipulations required to generate viable bone using autologous MSFs, as well as to further define acceptable carrier vehicles. The ability to proliferate in vitro and to differentiate in vivo to produce self-maintained bone that supports hematopoiesis makes MSFs promising candidates for bone regenerative cell therapy. In addition, osteogenesis by human MSFs will provide a viable in vivo model for human bone metabolism. Thus, this model system will be useful in manipulating conditions affecting bone homeostasis in an in vivo environment that cannot be otherwise created in humans.

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